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Integrin engagement modulates the phosphorylation of focal adhesion kinase, phagocytosis, and cell spreading in molluscan defence cells

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Abstract

Integrins play a key role in cellular immune responses in a variety of organisms; however, knowledge of integrins and their effects on cell signalling and functional responses in molluscan defence reactions is poor. Using integrin-mediated cell adhesion kits, $\alpha_V\beta_3$ and β_1 integrin-like subunits were identified on the surface of *Lymnaea stagnalis* haemocytes. Haemocyte binding via these integrins was found to be dependent on Ca^{2+}/Mg^{2+}. Western blotting with an anti-phospho (anti-active) focal adhesion kinase (FAK) antibody revealed a 120–125 kDa FAK-like protein in these cells; this protein was transiently phosphorylated upon haemocyte adhesion over 90 min, with maximal phosphorylation occurring after 30 min binding. Also, integrin engagement with the tetrapeptide Arg–Gly–Asp–Ser (RGDS) resulted in a rapid increase in phosphorylation of the FAK-like protein; however, RGDS did not affect the phosphorylation of extracellular signal-regulated kinase. Treatment of haemocytes with RGDS (2 mM) inhibited phagocytosis of *E. coli* bioparticles by 88%. Moreover, at this concentration, RGDS reduced cell spreading by 61%; stress fiber formation was also impaired. Taken together, these results demonstrate a role for integrins in *L. stagnalis* haemocyte adhesion and defence reactions and, for the first time, link integrin engagement to FAK activation in molluscs.

Keywords: Mollusc; Invertebrate defence; Haemocytes; FAK; ERK; RGDS

1. Introduction

Integrins are a family of heterodimeric, transmembrane adhesion receptors that play a critical role in cell–extracellular matrix interactions and in the organisation of the cytoskeleton [1,2]. Each heterodimer consists of a large extracellular domain, a single transmembrane segment and a relatively short cytoplasmic tail [3]. Upon integrin engagement, proteins that are involved in actin remodelling and are associated with focal adhesion sites become phosphorylated. One such protein is focal adhesion kinase (FAK), which associates with the protein tyrosine kinase Src. Although mechanisms of FAK activation are not fully understood, integrin clustering is known to result in autophosphorylation of FAK at Tyr^{397}; FAK then associates with the SH2 domain of Src family kinases [4]. Src subsequently phosphorylates FAK at Tyr^{925}, promotes recruitment of the Grb2–SH2 domain, and has been shown to trigger Ras-dependent activation of the mitogen-activated protein kinase (MAPK) pathway in NIH3T3 fibroblasts [5,6]. Src is then capable of phosphorylating paxillin and other proteins that are able to recruit various adaptor proteins [7,8].

Integrin subunits have been identified in many invertebrate cells [9–12], including haemocytes from the molluscs *Biomphalaria glabrata* [13] and *Crassostrea gigas* [14], and the activated form of FAK has been found in sea urchin embryos [15]. Studies have shown a link between integrin engagement and extracellular signal-regulated kinase (ERK) phosphorylation in NIH 3T3 fibroblasts and thyroid TAD-2 cells [16–18]. Furthermore, integrins and the FAK/Src complex have both been shown to play a role in phagocytosis of *E. coli* by insect haemocytes [19,20] and therefore seem to be important in the innate defence response of insects.

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Our knowledge of integrin-mediated processes in molluscan defence reactions is currently poor. Previously, our work on defence cells (haemocytes) of *Lymnaea stagnalis* revealed that ERK activity is necessary for efficient phagocytosis by these cells [21]. Using the integrin blocking tetrapeptide, Arg–Gly–Asp–Ser (RGDS), we now show that integrins are also important in the phagocytic response of molluscan haemocytes and that integrins facilitate haemocyte spreading. Furthermore, we demonstrate the presence of an *L. stagnalis* FAK-like protein, with an apparent molecular weight of 120–125 kDa, which is transiently phosphorylated upon haemocyte binding and RGDS engagement. This study therefore furthers our understanding of invertebrate integrin-mediated defence responses and is the first to explore the downstream signalling events that result from integrin engagement in molluscs.

2. Materials and methods

2.1. Materials

The anti-phospho p44/42 MAP kinase and anti-phospho FAK (Tyr^{925}) primary antibodies were purchased from Cell Signalling Technology (Beverly, USA), whereas the anti-phospho FAK (Tyr^{397}) antibody was from Calbiochem (Nottingham, UK). Hybond nitrocellulose membrane was from Amersham Biosciences (Amersham, UK). Horseradish peroxide-conjugated secondary antibody and the Opti-4CN detection kit were both from Bio-Rad (Hemel Hempstead, UK); the enhanced chemiluminescence (ECL) substrate kit and ECL film were from Perbio Science (Cheshire, UK). Both the $\alpha_V\beta_3$ and β_1 integrin-mediated cell adhesion kits were obtained from Chemicon International (Hampshire, UK). Vectashield was from Vector Laboratories (Burlingame, USA). The peptides Arg–Gly–Asp–Ser (RGDS) and Arg–Gly–Glu–Ser (RGES), cytochalasin D, anti-actin primary antibody, fluorescein isothiocyanate (FITC)-conjugated bioparticles, 4′-6-diamidino-2-phenylindole (DAPI), rhodamine phalloidin, and all other chemicals were from Sigma-Aldrich (Poole, UK).

2.2. Snails

Laboratory cultures of *L. stagnalis* were reared from eggs produced by adult snails purchased from Blades Biologicals (Edenbridge, UK). Juvenile snails were kept at room temperature until they reached a shell length of 20–30 mm. They were then transferred to an incubator and kept under a 12-h light–dark cycle at 20 °C. Tanks housing snails contained continuously aerated water, filtered through a Brimak/carbon filtration unit (Silverline Ltd., Winkleigh, UK). Water was changed weekly and snails were fed fresh lettuce ad libitum.

2.3. Haemolymph extraction and haemocyte treatments

Adult *L. stagnalis* were washed with distilled water and the haemolymph was extracted from each snail by head–foot retraction. Haemolymph was collected, pooled, and kept on ice in sterile snail saline (SSS [22]; 3 mM HEPES, 3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, pH 7.8, sterilised through a 0.22 μm disposable filter; 1 part SSS: 2 parts haemolymph). Haemocyte monolayers were then prepared by allowing cells to adhere to individual wells (200 μl diluted haemolymph per well) of a 24-well culture plate (Nunc) for 30 min at room temperature.

2.4. Integrin-mediated haemocyte adhesion assays

The integrin-mediated haemocyte adhesion assays were performed with integrin $\alpha_v\beta_3$-mediated and β_1-mediated cell adhesion kits using the manufacturer's recommended protocols. The kits utilise the antibody clones LM609 (for $\alpha_v\beta_3$ integrin) and P4G11 (for β_1 integrin), which target the functional binding region and calcium-dependent epitopes of these integrins, respectively. Briefly, the anti-integrin antibody-coated (experimental) and uncoated (control) strips were hydrated with 200 μl phosphate buffered saline (PBS with and without Ca^{2+}/Mg^{2+}) per well for 10 min at room temperature. The PBS was removed and 100 μl extracted haemolymph diluted in PBS (with and without Ca^{2+}/Mg^{2+}) was then added to each of the wells and incubated at 37 °C for 2 h. After incubation, diluted haemolymph was removed and each well was washed three times with 200 μl PBS (with and without Ca^{2+}/Mg^{2+}) before 100 μl of cell stain solution was added to each well and incubated for 5 min at room temperature. The stain was then removed and the wells were washed 5 times with PBS (with and without Ca^{2+}/Mg^{2+}) and allowed to air dry before the addition of 100 μl extraction buffer. Next, samples were incubated at room temperature on an orbital shaker until the stain had completely solubilised (5–10 min) enabling the absorbance (at 570 nm) of samples to be measured. The presence/absence of integrins and their requirement for Ca^{2+}/Mg^{2+} for adhesion in the assay were then determined by comparing the absorbance values of experimental wells with those of control wells.

HL60 (leukaemic) cells were used as a negative control. The HL60 cell line was grown in 20 ml culture flasks in RPMI 1640 medium, containing 10% foetal bovine serum, at 37 °C in an atmosphere containing 5% CO_2. Cells were passaged when densities reached 1×10^6 cells ml^{-1}. To harvest cells, the medium was transferred to a sterile tube and centrifuged for 5 min at 800×*g*. The cell pellet was then re-suspended in 1 ml PBS (containing Ca^{2+} and Mg^{2+}), and 100 μl of the cell suspension was placed in both experimental and control wells. Wells containing HL60 cells were then treated similarly to those containing haemocytes.

2.5. Phagocytosis and cell spreading

Haemocyte monolayers were prepared as previously described in individual wells of a 96-well culture plate (Nunc, 200 μl diluted haemolymph per well) and were washed three times with 200 μl SSS. Haemocytes were then incubated in RGDS (0–2 mM in SSS), RGES (control peptide, 0–2 mM in SSS), cytochalasin D (0–10 μM in methanol/SSS), or vehicle, for 30 min prior to the addition of FITC-conjugated bioparticles (6×10^6 per well). After haemocytes had been challenged with bioparticles for 1 h in a dark chamber at room temperature, in the presence of RGDS, RGES, cytochalasin D, or vehicle, bioparticles were removed and 2% (w/v) trypan blue was added to the wells for 2 min to quench extracellular fluorescence. Intracellular fluorescence was then quantified using a Fluorstar Optima microplate spectrofluorometer (BMG Labtech, Aylesbury, UK). Trypan blue dye exclusion assays were used to visually assess potentially lethal effects of the RGDS and RGES on haemocytes and neither peptide appeared to affect the viability of the haemocytes at the concentrations used.

To assess the effects of integrin blockade on haemocyte spreading, RGDS (2 mM) or RGES (2 mM) was added to freshly diluted haemolymph and was incubated with cells for 20 min on ice. Aliquots (100 μl) of treated haemolymph were then applied to glass coverslips and the haemocytes were left to bind for 30 min at room temperature. After brief washing with SSS to remove non-adherent haemocytes, the cells were immediately fixed and permeabilised in fixing buffer (3.7% (v/v) formaldehyde, 0.18% (v/v) Triton X100 in PBS) for 12 min. Coverslips were then incubated in rhodamine phalloidin (0.1 μg/ml) and DAPI (1 μg/ml) for 40 min before being mounted onto slides using Vectashield as the mounting medium. Cells were observed with a Zeiss Axiophot 20 photomicroscope using a triple filter; excitation wavelengths were 410, 505 and 585 nm (with beamsplitters: 395, 485 and 560 nm; and barriers: 460, 530 and 610 nm, respectively). Fields of view were randomly chosen and 120 images of individual cells from three separate experiments were digitally captured using a Nikon DN100 camera linked to Nikon Eclipse Net image analysis software. The area analysis function of this software was then used to determine the extent of cell spreading following different treatments.

2.6. Levels of phospho-FAK in bound and suspended cells

Haemocyte monolayers were prepared in 6-well culture plates (Nunc, 1 ml diluted haemolymph per well). Cells were left to bind to the plates for 30, 60 and 90 min before the haemolymph was removed and the haemocytes lysed with boiling SDS-PAGE sample buffer (62 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 5% (v/v) glycerol, 0.003% (w/v) bromophenol blue). The collected cell lysates were then transferred to microfuge tubes and sonicated for

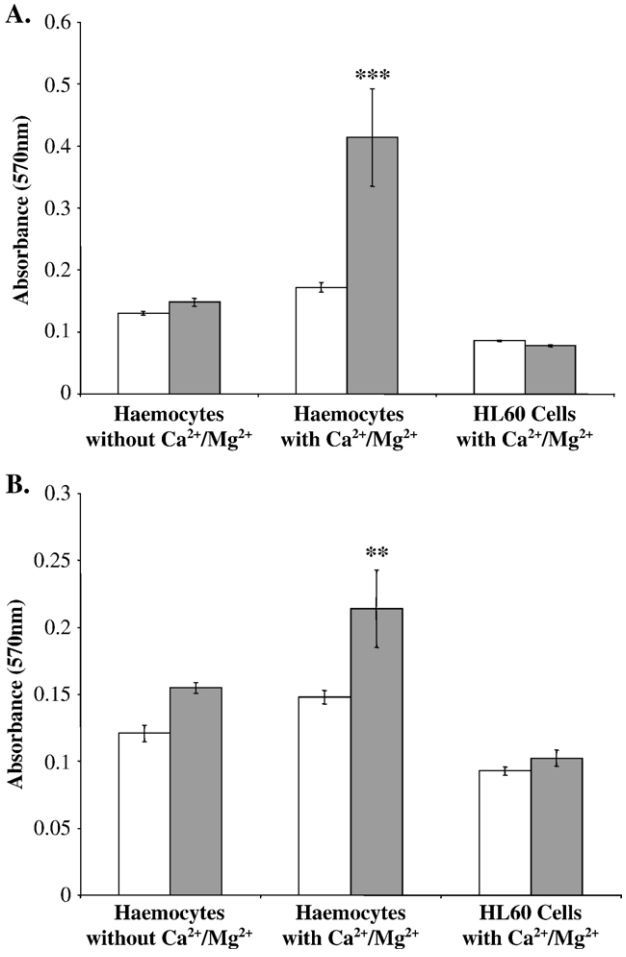

Fig. 1. Identification of integrin-like proteins on the surface of haemocytes. (A) $\alpha_V\beta_3$ integrin and (B) β_1 integrin binding assays were performed in the presence or absence of Ca^{2+}/Mg^{2+} using integrin-mediated cell adhesion kits as described in Materials and methods. The non-adherent HL60 cell line was used as an internal control. Wells of the test plates were either coated with the respective anti-integrin antibodies (experimental group, ■) or were not (control group, □). Values shown are mean absorbance at 570 nm (±S.E.M.; $n=7$); $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$, when compared to the control group for each treatment.

3 min before heating to 90 °C for a further 3 min. Cell lysates from unbound haemocytes were also prepared by centrifuging 1 ml of diluted haemolymph at 800×*g* for 10 min; the supernatant was then removed and the haemocyte pellet lysed with boiling SDS-PAGE sample buffer prior to sonication. Samples were then either electrophoresed immediately or stored at −20 °C.

2.7. The effect of RGDS on haemocyte phospho-ERK and phospho-FAK

Haemocyte monolayers were washed three times with 200 μl SSS after the 30 min binding period to remove dead and non-adherent cells. Monolayers were then left to equilibrate in SSS for 30 min prior to the addition of RGDS (2 mM) or RGES (2 mM) for various time periods (0–2 min for FAK activation and 0–30 min for ERK activation). At the appropriate time points, the SSS/RGDS or SSS/RGES was quickly removed and cells were lysed in boiling SDS-PAGE sample buffer. Cell lysates were then transferred to microfuge tubes, sonicated for 3 min and heated to 90 °C for a further 3 min prior to electrophoresis or storage at −20 °C.

2.8. SDS-PAGE and western analysis

Samples were loaded onto discontinuous SDS-PAGE slab gels, containing 10% acrylamide in the resolving gel. After electrophoresis, proteins were electroblotted onto Hybond nitrocellulose membrane (0.45 μm), using a semi-dry transfer unit, and homogeneous transfer was confirmed by staining with Ponceau S. Membranes were then blocked for 1 h at room temperature with 5% (w/v) non fat-dry milk in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 (TTBS). After blocking, membranes were incubated in anti-phospho-p44/42 MAPK (1:1000), anti-phospho FAK (Tyr^{925}) (1:900), or anti-phospho FAK (Tyr^{397}) (1:800) primary antibodies overnight at 4 °C; anti-actin antibodies (1:2500) were also employed to assess whether lanes had an equal loading of protein. After exposure to horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, signal was developed using either the Opti-4CN detection kit (for ERK and actin) or ECL reagent (for phospho-FAK). No signal was detected when membranes were incubated only in secondary antibody or normal rabbit IgG. The intensity of the immunoreactive signal was then analysed using Kodak 1D Image analysis software. Values were calculated as a net difference in phosphorylation with treatment compared to the control value for each replicate; thus, each control value was assigned a standardised value of 1.

2.9. Statistical analysis

Where appropriate, results were analysed with one-way analysis of variance (ANOVA) and post hoc multiple comparison tests (Tukey), using the statistical software package SPSS.

3. Results

3.1. Integrin-mediated haemocyte adhesion

To determine whether *L. stagnalis* haemocytes might use integrin-dependent mechanisms for binding extracellular substrates, integrin-mediated adhesion assays were carried out to evaluate the presence of integrins on the surface of haemocytes. Two anti-integrin antibody-based binding assays, which target the $\alpha_V\beta_3$ and β_1 integrins, were employed; for both assays, the control wells contained no antibody and the experimental wells were coated with the respective anti-integrin antibodies.

Results from the $\alpha_V\beta_3$ integrin assay show that *L. stagnalis* haemocytes possess $\alpha_V\beta_3$ integrin-like heterodimers. The experimental wells had a mean absorbance of 0.414, significantly greater than that of control wells with an absorbance of 0.172 ($P \leq 0.001$; Fig. 1A). Furthermore, in this assay, haemocytes required

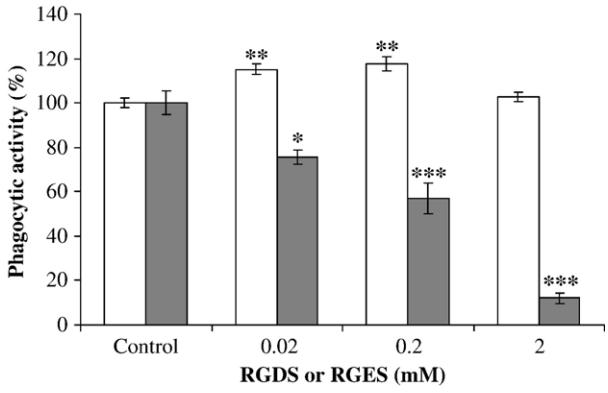

Fig. 2. Suppression of haemocyte phagocytic activity by RGDS (0.02–2 mM). Haemocytes were pre-incubated with RGDS (■), or RGES (□, control peptide), for 30 min before addition of fluorescent bioparticles; phagocytosis was then determined as described in Materials and methods. Values shown are mean phagocytic activity (±S.E.M.; $n=12$ for RGDS, $n=10$ for RGES); $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$, when compared with the untreated control group.

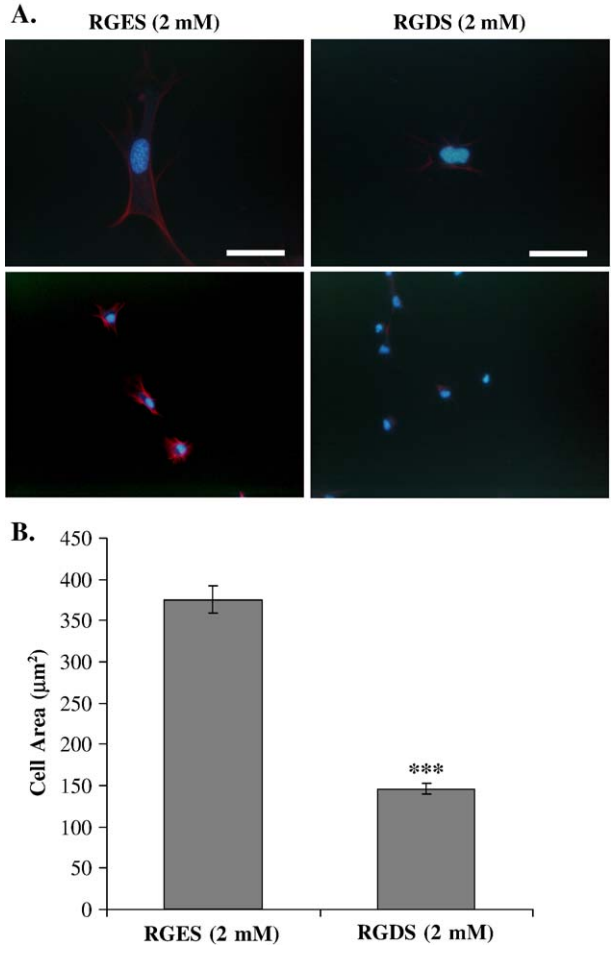

Fig. 3. Reduction of haemocyte cell spreading by RGDS (2 mM). Haemocytes were treated with RGDS, or RGES (control peptide), and their effect on haemocyte cell spreading on glass coverslips were determined following staining of haemocytes with rhodamine phalloidin (for F-actin) and DAPI (for nuclei) (A). Images of individual cells were captured and the area occupied by each cell determined using Nikon cell area analysis software (B); values shown are mean cell area (±S.E.M.; $n=60$ for each treatment), ***$P<0.001$. Bar=20 μm.

extracellular Ca^{2+} and Mg^{2+} for successful integrin engagement since significantly fewer cells were bound when these salts were absent ($P \leq 0.001$ when compared to experimental wells in the presence of salts; Fig. 1A). The non-adherent human leukeumic HL60 cell line was used as a negative control; lack of integrins on these cells resulted in low levels of adhesion, with control and experimental wells possessing mean absorbances of 0.08 and 0.07 respectively (Fig. 1A). In the assay for β_1 integrin, although the difference between the mean absorbance of the experimental wells (0.21) and control wells (0.15) in the presence of Ca^{2+}/Mg^{2+} was less than that observed in the $\alpha_V\beta_3$ assay, the difference was significant ($P \leq 0.01$; Fig. 1B); this finding is consistent with a β_1 integrin-like subunit being present on the haemocyte surface. Similar to $\alpha_V\beta_3$ integrin-mediated binding, haemocyte adhesion via the β_1 subunit appeared reliant on the presence of extracellular Ca^{2+}/Mg^{2+}, demonstrated by the significant reduction in binding in the absence of these salts ($P \leq 0.05$; Fig. 1B). HL60 cells were also utilised as a negative control in the β_1 integrin adhesion assay; the absorbance readings from HL60 control and experimental wells were low and were significantly less than seen with haemocytes in the presence of salts ($P \leq 0.001$).

3.2. Role of integrins in phagocytosis and cell spreading

To determine the role of haemocyte integrins in phagocytosis, cells were exposed to RGDS peptide (0.02–2 mM), control RGES peptide (0.02–2 mM), or vehicle, in the presence of *E. coli* bioparticles. Engagement of integrins with RGDS resulted in a dose-responsive reduction in phagocytic activity, with the greatest concentration (2 mM) reducing phagocytosis by 88%, when compared to vehicle controls ($P \leq 0.001$; Fig. 2). Lower RGDS concentrations also resulted in significant attenuation of bioparticle internalisation (reduced by 24% ($P \leq 0.05$) and 43% ($P \leq 0.001$) for 0.02 and 0.2 mM, respectively). Treatment of haemocytes with RGES control peptide (0.02 and 0.2 mM), slightly increased phagocytosis of bioparticles (Fig. 2).

Inhibition of haemocyte actin polymerisation with cytochalasin D (0.1–10 μM) also resulted in a dose-responsive reduction in

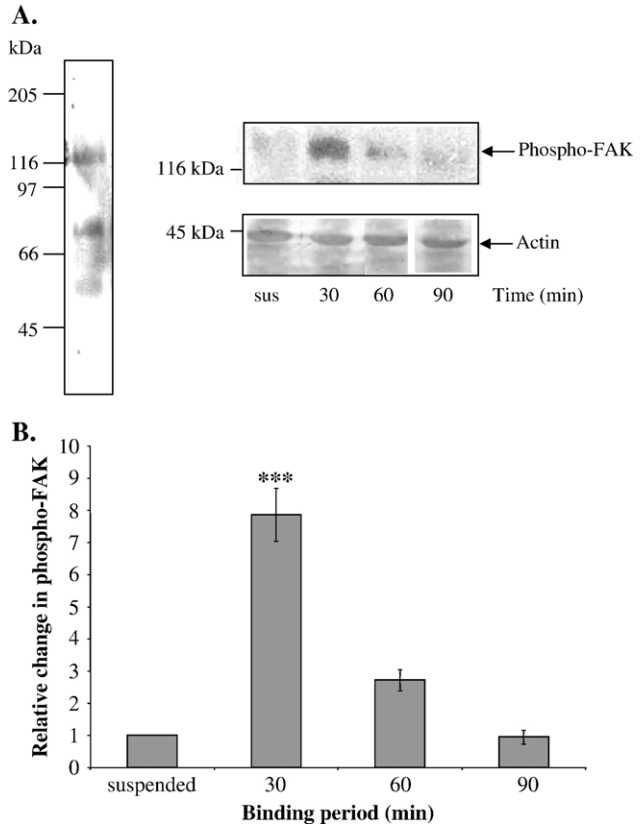

Fig. 4. Cell adhesion increases phosphorylation of a FAK-like protein in haemocytes. Cells were left to bind for 30, 60 or 90 min, or kept in suspension (sus). Proteins were extracted and subjected to western blotting with anti-phospho FAK (Tyr^{925}) and anti-actin antibodies as described in Materials and methods (A, right panel). The specificity of the anti-phospho FAK (Tyr^{925}) antibody towards the haemocyte FAK-like protein extracted from haemocytes after 30 min binding is shown in the left panel. Blots are representative of three independent experiments. Immunoreactive bands were analysed for their relative intensities and the mean fold-change in FAK phosphorylation calculated (B; ±S.E.M.; $n=3$); ***$P \leq 0.001$, when compared to suspended cells.

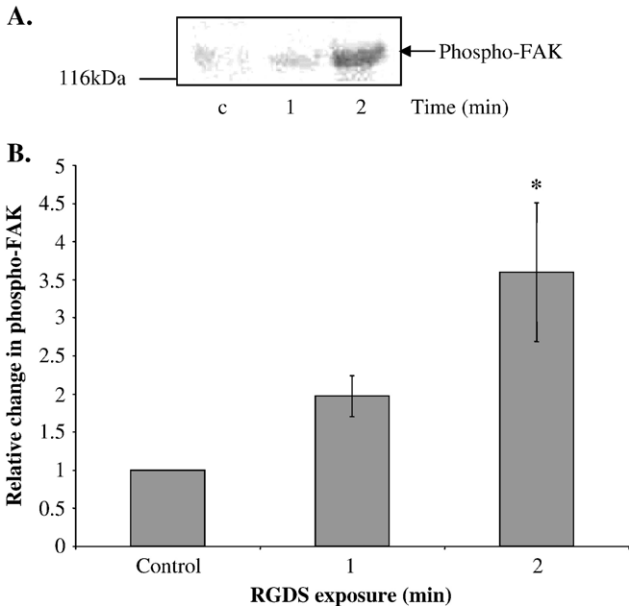

Fig. 5. Haemocytes exposed to RGDS possess increased FAK phosphorylation. Cells were either exposed to RGDS (2 mM) for 1 or 2 min, or vehicle (control, c). Proteins were extracted and subjected to Western blotting with anti-phospho FAK (Tyr^{925}) antibodies as described in Materials and methods (A). The blot shown is representative of three independent experiments. No changes in immunoreactivity were seen when haemocytes were exposed to the RGES (control) peptide, data not shown. Immunoreactive bands were analysed for their relative intensities and the mean fold-change in FAK phosphorylation calculated (B; ±S.E.M.; $n=3$); *$P \leq 0.05$, when compared to suspended cells.

phagocytosis of *E. coli* bioparticles by haemocytes ($P \leq 0.001$, data not shown). The highest dose of this inhibitor (10 μM) significantly suppressed phagocytic activity to 11% of the control ($P \leq 0.001$). Lower concentrations of this inhibitor also significantly reduced phagocytosis of bioparticles when compared to controls (reduced by 16% ($P \leq 0.01$) and 65% ($P \leq 0.001$) for 0.1 μM and 1 μM cytochalasin D, respectively).

Pre-treatment of haemolymph with RGDS (2 mM) for 20 min on ice did not appear to prevent haemocytes from subsequently forming stable contact with glass. However, in the presence of 2 mM RGDS, their ability to spread on glass coverslips was significantly impaired (Fig. 3A). Area analysis of individual cells revealed that, after 30 min binding, the mean area occupied by an individual haemocyte treated with RGDS was 61% less than that of a haemocyte exposed to control peptide (Fig. 3B). In addition, RGDS treatment appeared to have a marked effect on stress fiber formation in haemocytes, as evidenced by the consistent reduction in rhodamine phalloidin staining seen in cells exposed to this peptide (Fig. 3A). The spread of cells exposed to RGES was found to be similar to that of cell exposed to SSS alone (data not shown).

3.3. Haemocyte binding and phosphorylation of FAK

The anti-phospho FAK (Tyr^{925}) antibody detected an immunoreactive protein of approximately 120–125 kDa in extracts of adhered haemocytes (Fig. 4A), but no signal was observed when the anti-phospho FAK (Tyr^{397}) antibody was used (data not shown). The FAK-like protein detected appeared to be maximally phosphorylated at 30 min post-binding, whereas phosphorylated FAK in suspended cells was virtually undetectable by Western analyses (Fig. 4A). When bands were analysed for their relative intensities, cells that had been allowed to bind for 30 min had approximately 8 times more phosphorylated FAK-like protein than suspended cells ($P \leq 0.001$; Fig. 4B). The phosphorylation of the FAK-like protein upon binding appeared to be transient, with phosphorylation levels being reduced to approximately three times those of suspended cells after 60 min and returning to levels similar to these cells after 90 min (Fig. 4B).

3.4. Effect of RGDS on FAK and ERK phosphorylation

To assess whether integrin engagement by RGDS (2 mM), results in altered phosphorylation of the FAK-like protein in haemocytes, RGDS or RGES was delivered to haemocytes after a 60 min equilibration period. This equilibration period allowed FAK phosphorylation to return to near basal levels as previously determined (Fig. 4). Western analyses demonstrated that RGDS treatment resulted in increased phosphorylation of the haemocyte FAK-like protein over 2 min (Fig. 5A). Analysis of the band intensities revealed that phosphorylation levels increased 2-fold after 1 min exposure to RGDS, and approximately 3.5-fold after 2 min ($P \leq 0.05$; Fig. 5B). RGES treatment did not increase FAK phosphorylation levels at any of the time points studied, and only a very weak immunoreactive signal was obtained (data not shown). In contrast to FAK phosphorylation, ERK phosphorylation levels

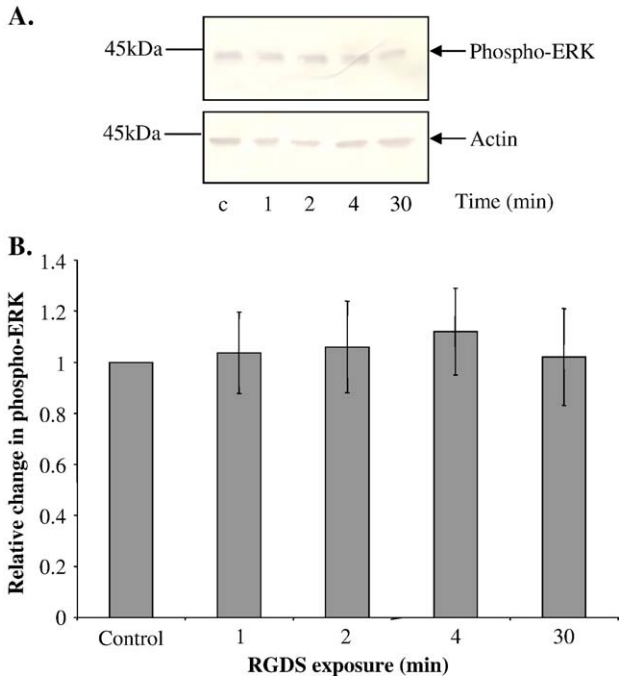

Fig. 6. RGDS exposure does not result in increased haemocyte ERK activity. Haemocytes were exposed to RGDS for 1, 2, 4, and 30 min, or vehicle (control; c). Proteins were extracted and subjected to western blotting with anti-phospho p42/44 MAPK and anti-actin antibodies as described in Materials and methods (A). Blots shown are representative of three independent experiments. Immunoreactive bands were analysed for their relative intensities and the mean fold-change in ERK phosphorylation calculated (B; ±S.E.M.; $n=3$).

did not change significantly when haemocytes were exposed to RGDS, for short (up to 4 min) or longer (30 min) periods (Fig. 6).

4. Discussion

The FAK/Src complex has been shown to play a role in phagocytosis by insect haemocytes [20], and an activated form of FAK has been identified in sea urchin embryos [15] using anti-phospho-FAK antibodies. Although integrins have also been identified in many other invertebrate cells [9–12], our knowledge of molluscan integrins is relatively poor. While a β_1 integrin-like protein has been successfully cloned from an embryonic cell line (Bge) derived from the gastropod mollusc *B. glabrata* [13] and haemocytes from the bivalve mollusc *Crassostrea gigas* [14], there have been no previous reports linking integrin engagement to intracellular signalling events in molluscs.

Integrins play a key role in cell adhesion, and as such are particularly important in immune cell function. With the aid of integrin-based cell adhesion kits, *L. stagnalis* haemocytes were found to possess $\alpha_V\beta_3$ and β_1 integrin-like proteins. Because the antibodies used in these kits target the functional binding region and calcium-dependent epitopes on $\alpha_V\beta_3$ and β_1 integrins, respectively, experiments were performed to assess the Ca^{2+}/Mg^{2+} dependency of integrin binding. Since adhesion of haemocytes was found to be impaired when these cations were omitted from the medium, Ca^{2+}/Mg^{2+} appear to be important for binding by these integrins in *L. stagnalis* haemocytes. Given that integrin clustering is known to activate FAK in mammalian cells [23], we set out to determine levels of phosphorylated FAK in both suspended and adherent haemocytes using an anti-phospho FAK (Tyr^{925}) antibody. Activation of FAK has been reported in skeletal muscle cells upon binding to fibrinogen coated plates [24], and cell spreading and migration are increased by expressing FAK in FAK-null mouse embryo cells [25]. Moreover, when expressed in NIH3T3 cells, the *Drosophila* homologue of vertebrate FAK, DFak56, localises to focal contacts and displays increased phosphotyrosine content in response to plating on fibronectin [26]. In the present study, the FAK-like protein in haemocytes left to bind to plastic for 30 min had an 8-fold increase in phosphorylation compared with suspended cells. The phosphorylation of this molluscan FAK-like protein at a region similar to Tyr^{925} of vertebrate FAK was transient, with phosphorylation reduced to near basal levels after 90 min of binding. Moreover, haemocytes in suspension lacked significantly detectable amounts of the phosphorylated FAK-like protein, suggesting that FAK activity is reliant on cell adhesion in haemocytes. Similar results were reported by Schlaepfer et al. [8], who demonstrated tyrosine phosphorylated FAK in fibroblasts that were bound to fibronectin, but not in those that were suspended. Cells cultured on fibronectin-coated plates possessed FAK activity 20 min after plating, with activation occurring for up to 6 h [8]. Integrin-specific adhesion of NIH 3T3 cells is known to activate all components of the ERK pathway (Raf-1, MEK and ERK) for up to 1 h [27]. We have previously found that ERK activity is up-regulated after 30 min when *L. stagnalis* haemocytes are bound to plastic, although unlike FAK, activity levels are not substantially reduced over 90 min (unpublished data).

Using an anti-phospho-FAK (Tyr^{397}) antibody, Metheniti et al. [20] demonstrated a specific role for the FAK/Src complex in phagocytosis by insect haemocytes, with FAK being phosphorylated in the presence of *E. coli*. In the present study, the anti-phospho Tyr^{397} antibody failed to detect haemocyte proteins suggesting that the autophosphorylation site in the *L. stagnalis* FAK-like protein is insufficiently homologous to that of mammalian or insect FAK. Since both integrins and FAK have shown to be involved in the phagocytic activity of insect haemocytes [19,20], phagocytosis assays were performed with RGDS. RGDS is an integrin blocking tetrapeptide that inhibits phagocytosis in alveolar macrophages [28] and fish haemostatic cells [29]. RGDS also blocks integrin signalling in RAW cells [30]. In the present study, RGDS reduced phagocytic activity of haemocytes significantly, demonstrating the involvement of integrin receptors in this process. Moreover, blockade of actin remodelling with cytochalasin D resulted in a similar effect, demonstrating a crucial role for the actin cytoskeleton in haemocyte defence. At similar concentrations to those used in this study, cytochalasin D has been shown to inhibit FAK activity in NIH 3T3 cells [17]; such inhibition of FAK can result in amplified signalling to ERK via Shc [31].

Although RGDS did not appear to affect the ability of *L. stagnalis* haemocytes to form stable contact with glass, this integrin-specific adhesion inhibitor did influence the spreading behaviour of haemocytes, reducing cell spread by 61% when compared to haemocytes treated with RGES. Thus, integrins appear to play an important role in certain haemocyte attachment events. Spreading of haemocytes derived from the snail *B. glabrata* has also been shown to be inhibited by the RGDS peptide, with 2 mM RGDS reducing the number of haemocytes designated as 'spread' by approximately 90% [32]. Moreover, working with the embryonic Bge cell line, Humphries et al. [33] demonstrated a role for PKC and Ras-like proteins in cell spreading. In the present study, staining of haemocytes with rhodamine phalloidin also enabled visualisation of stress fibers, and RGDS was found to significantly inhibit their formation; this finding is likely a consequence of less focal adhesion sites being present.

Western analysis showed that RGDS binding to adherent haemocytes resulted in the rapid phosphorylation of the haemocyte FAK-like protein on Tyr as early as 1 min after exposure. Interestingly, however, no increases in haemocyte ERK activity were detected following exposure to this peptide. Integrin engagement has previously been linked to ERK activation in NIH 3T3 and thyroid TAD-2 cells [16–18], and FAK activation can stimulate ERK in mouse fibroblast cells [16]. However, integrin-mediated activation of MAP kinase can be also independent of FAK in fibroblasts [34]. Furthermore, integrin-mediated activation of FAK in these cells is not necessarily required for signalling to ERK following growth factor stimulation [31]. In contrast, in NIH 3T3 cells integrin-mediated activation of MAP kinase can occur, but this happens via a pathway independent of Ras [27]. FAK over-expression can enhance fibronectin stimulated activation of ERK by

approximately 4-fold in human 293 cells, where FAK-mediated association and activation of c-Src appears essential for maximal signalling to ERK 2 [35]. Although the mechanisms for ERK activation following integrin clustering seem complex, the results obtained in the current study imply that integrin/FAK downstream signalling might be Ras-independent, as demonstrated in fibroblasts, because ERK activity is not up-regulated following integrin engagement by RGDS. However, further work is necessary to help delineate these events in *L. stagnalis* haemocytes.

Taken together, our results reveal the presence of integrin-like molecules in *L. stagnalis* haemocytes, show that they play a key role in phagocytosis and cell spreading, and demonstrate that integrin engagement results in the phosphorylation of a FAK-like protein in these cells. Clearly, it would be interesting to identify the downstream targets of FAK in *L. stagnalis* haemocytes, and other proteins recruited to the focal adhesion sites following integrin engagement. In mammalian systems, FAK activity can be reliant on Ras [36], and intracellular calcium and PKC activation when cells are stimulated with collagen [37]. Moreover, PKC has been implicated in integrin-mediated spreading of muscle cells [24], and can act as a key intermediary in integrin signalling to FAK in many cell types [38–40]. Phosphatidylinositol-3-kinase is also involved in FAK-independent signalling from the β_1 integrin subunit [41]. It would be interesting to determine whether these pathways coordinate integrin-mediated cell signalling events in *L. stagnalis* haemocytes; the work with Bge cells that shows inhibition of spreading by PKC and Ras inhibitors [33] suggests that multiple signalling events link integrin engagement to downstream functional responses. Work with *Drosophila* has clearly identified a molecular role for integrins [42] and cytoskeletal regulatory proteins in various cellular processes, exemplified by D-SCAR and profilin in phagocytosis [43], and DFak56 in cell migration [26]. Further work on molluscan integrins and dissection of the signal transduction pathways that are affected by integrin engagement in molluscan haemocytes will ultimately facilitate our understanding of haemocyte survival, gene transcription and cell migration, as well as enhancing our knowledge of the molecular events regulating phagocytosis in molluscs.

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